



## Review

## Importance of nucleotide sequence and chemical modifications of antisense oligonucleotides

Sudhir Agrawal \*

Hybridon, Inc., 155 Fortune Blvd., Milford, MA 01757, USA

Received 22 February 1999; received in revised form 15 June 1999; accepted 15 June 1999

## Abstract

The antisense approach is conceptually simple and elegant; to design an inhibitor of a specific mRNA, one needs only to know the sequence of the targeted mRNA and an appropriately modified complementary oligonucleotide. Of the many analogs of oligodeoxynucleotides explored as antisense agents, phosphorothioate analogs have been studied the most extensively. The use of phosphorothioate oligodeoxynucleotides as antisense agents in various studies have shown promising results. However, they have also indicated that quite often, biological effects observed could be solely or partly non-specific in nature. It is becoming clear that not all phosphorothioate oligodeoxynucleotides of varying length and base composition are the same, and important consideration should be given to maintain antisense mechanisms while identifying effective antisense oligonucleotides. In this review, I have summarized the progress made in my laboratory in understanding the specificity and mechanism of actions of phosphorothioate oligonucleotides and the rationale for designing second-generation mixed-backbone oligonucleotides. © 1999 Elsevier Science B.V. All rights reserved.

**Keywords:** Antisense; Oligonucleotide; Mixed-backbone oligonucleotide; Pharmacokinetics; Immune stimulation; CpG; Pro-drug; Antiviral

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\* Fax: +1-508-482-7692; E-mail: sagrawal@hybridon.com

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## 1. Introduction

Antisense oligonucleotides provide a rationally designed tool to manipulate expression of specific gene products [1,2]. In the last 10 years, antisense oligonucleotides have been widely used in various *in vitro* and *in vivo* models [3–11]. Based on the promising results obtained, antisense oligonucleotides are being explored for their potential as therapeutic agents for the treatment of viral infections, cancers and inflammatory disorders [5,6,8,9]. The antisense approach is conceptually simple; to design an inhibitor, one needs only to know the sequence of the targeted gene product (mRNA) and to identify any specific modifications of the oligonucleotide. Antisense oligonucleotides have been used extensively in the last several years, with mixed results. [8,10–12]. The most-studied of the oligodeoxynucleotides are the phosphorothioate analogs (PS-oligos), in which one of the non-bridging oxygen atoms in the phosphate backbone is replaced by a sulfur [3–11]. Based on the results obtained to date with PS-oligos, it has become evident that PS-oligos exert biological activity by multiple mechanisms of actions [8–13]. The mechanisms of action can be classified into three categories: (a) sequence-specific activity by binding to mRNA, referred here as antisense activity; (b) sequence-specific activity by interacting with other factors than mRNA, referred here as non-antisense ac-

tivity; and (c) non-sequence-specific activity. In this review, I have attempted to summarize the progress in my laboratory in understanding the rules that govern the specificity and mechanisms of actions of PS-oligos and the rationale for designing second-generation antisense oligonucleotides. A number of other investigators have published their results and views, which also appear in this issue.

## 2. Phosphorothioate oligodeoxynucleotides

PS-oligos have a negatively charged backbone and are capable of supporting RNase-H activity similar to phosphodiester oligodeoxynucleotides, but PS-oligos have greater resistance to nuclease degradation than do phosphodiesters. These intrinsic properties have made PS-oligos the choice as first-generation antisense oligonucleotides [3-11].

We and others initially used PS-oligos as inhibitors of HIV-1 replication in HIV-1-infected cells [14–23]. From the results obtained it was obvious that the PS-oligos effectively inhibited HIV-1 replication, but the apparent mechanism of HIV-1 inhibition differed depending on the experimental model [14–22]. PS-Oligos inhibited HIV-1 replication by antisense mechanisms as well as non-sequence-specific mechanisms [14–25]. The non-sequence-specific mechanism was most probably due to the polyanionic nature of PS-

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In the last six years, in which PS-oligos have been used to study expression of genes in cancer and in other tissues, it is suggested that the use of PS-oligos was primarily to study the function of MDM2 oncogene. MDM2 encodes for an Rb-like protein that can bind to p53 and repress its transcription [38,39]. In the presence of PS-oligos, MDM2 and protein levels were reduced and protein levels led to increased p53 transcription and cell growth [37]. The effect of PS-oligos on the control PS-oligos was also shown to show that active

It has been shown that CpG oligos with certain properties in immune stimulation contain a CpG motif and a 5'-phosphate [40–46]. PS-Oligos are able to induce cytokines and gamma-IFN [47]. These cytokines induce antiviral activities [47,48].

### 3. Pharmacokin

Pharmacokinetics of intravenous administration of the plasma from 30 min to highly perfused marrow, and in other tissues [5] was in urine, v

oligos [14–25]. Similar results were observed with PS-oligos used to inhibit replication of influenza virus and other viruses [26]. In some recent studies, the biological activity observed with PS-oligos has also been associated with their polyanionic nature [27,28].

In the last six years, there have been many reports in which PS-oligos have been used to inhibit over-expression of cellular gene products implicated in cancer and inflammation. The results strongly suggest that the inhibition observed in these studies was primarily due to antisense activity [3–11,30–36]. We recently used PS-oligos complementary to an MDM2 oncogene [37]. The MDM2 oncogene encodes for an inhibitor of the p53 tumor suppressor protein that regulates p53 in a negative feedback loop [38,39]. In this study, selected antisense PS-oligos inhibited MDM2 expression at both the mRNA and protein levels [37]. Suppression of MDM2 oncoprotein led to a decrease in MDM2-p53 complex formation, which in turn resulted in an increase in p53 transcriptional activity, and finally to apoptosis [37]. The effects observed were sequence specific, as the control PS-oligos (with four mismatches) did not show such activity.

It has been shown in a number of studies that PS-oligos with CpG motifs have immune-stimulatory properties in rodents [11,40–46]. The severity of immune stimulation depends on the position of the CpG motif and its flanking sequence of PS-oligos [40–46]. PS-Oligos containing CpG motifs are known to induce cytokines, including IL-6, IL-12, TNF- $\alpha$ , gamma-IFN [43,44], and also chemokines [45]. These cytokines induced by PS-oligos containing CpG motifs have been shown directly or indirectly to have antiviral [47,48], anticancer [49], and antibacterial activities [50].

### 3. Pharmacokinetics and tissue distribution

Pharmacokinetics of PS-oligos in mice following intravenous administration showed rapid elimination from the plasma compartment with half-lives ranging from 30 min to 1 h [51]. PS-Oligo was distributed to highly perfused organs, such as kidney, liver, bone marrow, and spleen, in higher concentration than other tissues [51]. The primary route of elimination was in urine, with smaller amounts found in feces

[51]. Following intraperitoneal or subcutaneous administration, no significant differences in tissue distribution were observed, except that lower maximum plasma concentrations were achieved than with intravenous administration [51,52]. The analysis of extracted PS-oligos from plasma and tissues showed the presence of both intact and degraded forms of the PS-oligo [51,52]. Protection of PS-oligos on the 3'-end significantly minimized degradation, this suggested that *in vivo* degradation was primarily due to 3'-exo-nucleases [53]. Detailed analysis of the extracted oligo showed that the PS-oligo was degraded primarily from the 3'-end, but some degradation products were generated following degradation from the 5'-end and from both the 3'- and 5'-ends [54]. Similar pharmacokinetic and tissue disposition results were obtained in rats [55] and monkeys [56]. In humans, the plasma pharmacokinetic profile and elimination in urine were similar to those observed in monkeys [57]. Similar results, in general, have been reported with PS-oligos of varying sequences [58]. The pharmacokinetics of PS-oligos are found to be largely sequence independent except for PS-oligos that can form hyperstructures (e.g., G-rich oligos) [59].

### 4. Safety

Safety studies of PS-oligos in mice and rats show sequence-dependent side effects [10,11]. These side effects include splenomegaly, thrombocytopenia, and elevation of the liver enzymes, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) [10,11,60]. Histopathological examinations have revealed multi-organ mononuclear cells infiltrates, reticuloendothelial cell and lymphoid hyperplasia, and renal tubule degeneration [60]. The severity of these side effects are dependent on the dose and the frequency and duration of administration [60]. Similar results have been reported with other PS-oligos [61].

The safety profile of PS-oligos in monkeys is different from that observed in mice and rats [62]. Intravenous administration of PS-oligos in monkeys caused a brief increase, followed by a prolonged decrease, in arterial blood pressure, and a transient decrease in peripheral total white blood cells and

*Properties essential in an oligonucleotide for antisense activity*

- Nuclease stability
- Cellular uptake
- Specificity for target RNA
- Hybridization by Watson Crick base pairing
- Affinity for target RNA
- RNase H activation

**Phosphorothioate Oligodeoxynucleotide**

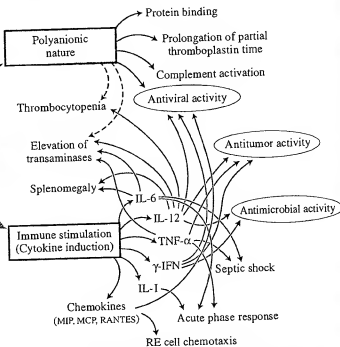


Fig. 1. Factors affecting the mechanism of action of oligonucleotides. Antisense oligonucleotides are designed to bind to targeted mRNA and inhibit translation. An effective oligonucleotide should be stable to nucleases and taken up by cells. In addition, oligonucleotides should have strong affinity for the target mRNA and should activate RNase H for RNA cleavage. Based on the nucleotide sequence and the nature of the internucleotide linkages, however, oligonucleotides display additional properties that may interfere with the specificity and mechanism of action of the oligonucleotide. Two major properties are the polyanionic nature and immune stimulation. As shown above in a generalized way, the polyanionic nature and immune stimulation may produce biological activities and side effects that compromise the specificity and action of a given oligonucleotide. Minimization of polyanionic nature and immune stimulatory properties by appropriate chemical modification should be considered in second-generation oligonucleotides.

neutrophil counts [62]. In addition, complement activation and prolongation of aPTT have also been observed [62]. These side effects are sequence independent, however, dependent on the concentration of PS-oligo in the plasma compartment, and can be minimized by slow intravenous infusion [62]. A similar observation has been made with other PS-oligos [63]. PS-Oligos that can form hyperstructures (e.g., G-rich oligos) have greater effects on complement activation and prolongation of aPTT [64].

A PS-oligo (GEM 91) has been administered to humans by 2- or 24-h intravenous infusion. The side effects observed included thrombocytopenia, elevation of transaminases, and prolongation of aPTT [65]. These side effects were dependent on the dose and duration of the treatment and the frequency of administration. Administration of lower doses of PS-oligos had minimal changes in the above mentioned side effects [65–67].

## 5. Understanding of PS-oligos

On the basis of knowledge gained to date with PS-oligos, the following generalizations can be made.

1. PS-oligos have built-in properties (e.g., affinity for target mRNA, nuclease stability, cellular uptake, and RNase H activation) required for antisense activity (Fig. 1).
2. PS-oligos behave like polyanionic molecules as is evident from the inhibitory activity observed in viral replication assays, binding to growth factors, serum proteins, etc. In addition, activation of complement and prolongation of aPTT in monkeys and humans is associated with the polyanionic characteristics of PS-oligos (Fig. 1).
3. PS-oligos have sequence-specific biological activities, which are not due to hybridization to specific mRNA. Two types of mechanisms may be in-

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## 6. Beyond

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## 7. Minimiz

The poly been assoc sequence-spe 28] and also gation of a phorothioa 2'-O-methy thioate 2', that the po what differ [73]. These ment activa

involved: immune stimulation (Fig. 1) and binding of factors (e.g., transcription factor and proteins) to a decoy or specific motif of PS-oligos [67-69].

## 6. Beyond PS-oligos

The use of PS-oligos in various studies has shown that antisense oligonucleotides can be used to selectively inhibit the expression of targeted mRNA in cell culture and in vivo [3-11,29-36]. Certain undesirable properties (e.g., polyanionic nature, immune stimulation) limit their potential for wider use as therapeutic agents and also as tools in elucidating the function of specific target genes (Fig. 1).

To overcome some of the limitations of PS-oligos, extensive efforts have been made to synthesize various analogs of oligonucleotides [3-11,70]. These analogs include deoxynucleotides with modification of the internucleotide linkages, heterocyclic bases, or sugar [3-11,70]. Some of these analogs have higher affinity for target RNA and increased resistance to serum and cellular nucleases than PS-oligos. Studies of these analogs in various cell culture models have not yielded encouraging results compared with those obtained with PS-oligos.

In the last few years, we have made attempts to rationally minimize the undesirable properties of PS-oligos, while maintaining the properties essential for antisense activity (Fig. 1). The following are two examples in this respect.

## 7. Minimization of polyanionic nature of PS-oligos

The polyanionic characteristics of PS-oligos have been associated with some of the observed non-sequence-specific biological activities [14,15,21,23,25-28] and also with complement activation and prolongation of aPTT [62,71,72]. In our studies using phosphorothioate oligoribonucleotides, phosphorothioate 2'-O-methyloligoribonucleotides, and phosphorothioate 2',5'-oligoribonucleotides, it became clear that the polyanionic nature of these analogs is somewhat different from that observed with PS-oligos [73]. These analogs had less of an effect on complement activation and prolongation of aPTT, suggest-

ing that not only the phosphorothioate linkage also the nature of the nucleosides of PS-oligo responsible for the polyanionic characteristics. It is possible that Rp and Sp diastereomers of PS-c have different polyanionic characteristics; one of the isomers of PS-oligoribonucleotides has less pronounced polyanionic characteristics because of 2'-hydroxyl group or other 2'-modifications.

There have been no reports on the polyan characteristics of Rp and Sp stereospecific PS-oligos. We have observed that Rp stereospecific PS-c have a greater affinity for complementary DNA and less stability towards nucleases than do Sp stereospecific PS-oligos [74]. Also, Rp PS-oligos were better substrates for RNase H than were stereospecific PS-oligos [74]. Independent studies using Sp stereospecific PS-oligos have shown that they have less affinity for complementary RNA and greater stability towards nucleases than do Rp and Sp stereospecific PS-oligos [75]. Lack of efficient synthesis methodologies to obtain stereospecific Rp or Sp PS-oligos have been preventing us from performing detailed studies. Recently, however, we have succeeded in synthesizing stereo-enriched Rp and Sp PS-oligos and also PS-oligos with an appropriate mix of Rp and Sp linkages [76,77]; detailed studies are now under way.

To minimize the polyanionic nature of the oligos, we have taken advantage of the reduced anionic characteristics of 2'-O-methylribonucleosides, (as observed by reduced complement activation and prolongation of aPTT), and have substituted few deoxynucleosides with 2'-O-methylribonucleosides, either at the 3'-end or both the 3'-ends or in the center of the PS-oligos [7] (Fig. 2). The overall result of this substitution increased affinity to target RNA, stability towards nucleases, and reduced polyanionic-related effects. In addition, these oligonucleotides retained the RNase H activating capability due to the presence of PS-oligo [78-80]. Similar results have been obtained by incorporating other modified oligonucleotides [81-83]. In addition to chemical modifications, polyanionic related effects can be minimized with the use of appropriate formulations. Urea protamine has been shown to minimize these effects [72].

Table 1  
Structure and sequence of oligonucleotides

1. TCG TCG CTG TCT CCG CTT CTT CTT GCC
2. <sup>me</sup>TCG <sup>me</sup>TCG CTG TCT CCG CTT CTT CTT GCC
3. TCG TCG CTG TCT CCG CTT CTT CTT GCC
4. TCG TCG CTG TCT CCG CTT CTT CTT GCC
5. CTC TCG CAC CCA TCT CTC TCC TTC T
6. CTC TCG CAC CCA TCT CTC TCC TTC T
7. CTC TCG CAC CCA TCT CTC TCC TTC T
8. CTC TCG CAC CCA TCT CTC TCC TTC T
9. CCG CAC CCA TCT CTC TCC UUC U
10. GCG TGC CTC CTC ACT GGC
11. CGC CGG GAT CTC GAT GCT CAT
12. CCG CTC TTC CTC ACT GGT
13. CCG UGC CTC CTC AUC GGC
14. CTC TCG CAC CCA TCT CTC TCC TTC T
15. CTC TCG CAC CCA UCU CTC TCC TTC T
16. CTC TCG CAC CCA UCU CTC TCC TTC T

All sequences are phosphorothioate; C - 5-methylcytosine;  
X-X - methylphosphonate linkage; XX - 2'-O-methyl ribonucleoside;  
XX - 2'-O-methylribonucleoside with phosphodiester linkages.

Oligo 1 is complementary to *rev* gene of HIV-1; oligo 5 is complementary to *gag* gene of HIV-1; oligo 10 is complementary to R1 $\alpha$  subunit of human protein kinase A (PKA); oligo 11 is complementary to RII $\beta$  subunit of human PKA; oligo 12 is complementary to R1 $\alpha$  subunit of mouse PKA.

## 8. Minimization of immune stimulation by PS-oligos

PS-oligos containing a CpG motif and the appropriate flanking sequences are known to be immune stimulatory [11,40–46]. Recent studies have indicated that activation of immune stimulation is through induction of mitogen-activated protein kinases [84,85].

The immune stimulation by CpG-containing PS-oligos results in induction of various cytokines, which has a therapeutic effect; these PS-oligos are being developed as novel therapeutic agents [47–50]. At the same time, these cytokines at higher doses have been associated to side effects [45].

We have studied PS-oligos for their safety profile in mice and rats and have reached the conclusion that the presence of CpG motif in PS-oligos adds to the severity of the toxicity observed [11]. For example, oligo 1 (Table 1) administered to mice causes thrombocytopenia, elevation of transaminases, and enlargement of the spleen (Fig. 3A). In addition, histopathological changes in the kidney, liver, and spleen were also noted. Modification of the CpG motif significantly minimized the side effects observed with oligo 1; these modifications included: replacement of the cytosine of the CpG motif with a 5'-methyl cytosine (oligo 2, Table 1); replacement of the phosphorothioate linkage of the CpG motif with a methylphosphonate linkage (oligo 3, Table 1); and replacement of the entire CpG motif with 2'-O-methylribonucleosides (oligo 4, Table 1) (Fig. 3A). Minimization of the histopathological changes was also noted with oligos 2, 3, and 4. These modifications suppressed the immune stimulatory properties of the CpG motif [42]. Reduction in the toxicity of oligo 1 produced by these modifications strongly suggests that PS-oligos have similar toxicities, but the severity of toxicity is increased due to presence of CpG motif [11] and their immune-stimulatory properties. Similar results were observed in rats with oligos 5, 6, 7, and 8 (Fig. 3B).

It is important to note that the flanking sequence of the CpG motif is a major factor in inducing immune stimulation, and not all PS-oligos with the CpG motif will behave in the same manner [11,41]. Oligos 10, 11, and 12, which contain the CpG motif at different positions in their sequences and flanking sequences, had significantly different toxicity profiles in mice (Fig. 3C). Oligo 10 showed more toxicity than oligo 11 and 12. It is also important to note that immune stimulation due to a given PS-oligo sequence depends on the host, the dose, and the route of administration (Q. Zhao, S. Agrawal, unpublished data).

The above discussion and results suggest that a given PS-oligo can be appropriately modified to sup-

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## 9. Mixed-

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## PS-Oligo

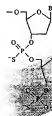


Fig. 2. Struc  
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MBOs refer  
corporation

aining PS-oligonucleotides, which are being used [47–50]. At lower doses have

a safety profile like the conclusion that PS-oligos add little [11]. For example, to mice causes aminases, and in addition, histology, liver, and one of the CpG de effects observed included: pG motif with a pG motif with the CpG motif (Table 1) (Fig. 4). These modulatory properties in the toxicity studies strongly suggest that the presence of immunostimulatory properties in rats with oli-

gating sequence in inducing immunostimulatory oligos with the same manner [11,41]. In the CpG motif studies and flanking toxicity profiles did more toxicity. It is important to note that given PS-oligos at a dose, and the S. Agrawal, un-

studies suggest that a modified to sup-

press its immune-stimulatory properties and the resulting toxicity. Furthermore, these modifications can be made more rationally to also improve general therapeutic potential of oligonucleotides as discussed below.

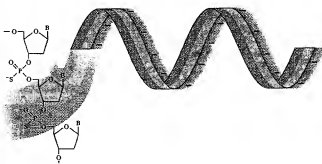
## 9. Mixed-backbone oligonucleotides (MBOs)

As it is evident from the above discussion, the nucleotide composition and nature of the nucleotide and internucleotide linkages alone or in combination dictate the biophysical, biochemical, and biological properties of oligonucleotides [70]. A number of oligonucleotide analogs have been studied that display properties different from those of PS-oligos in terms of resistance to nucleases, affinity to target RNA, cellular uptake, activation of RNase H, and more importantly, the in vivo pharmacokinetic profile [3–

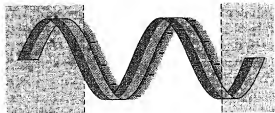
11]. In our earlier studies, we employed phosphorothioate oligoribonucleotides which bind to RNase H with higher affinity than PS-oligos, but do not activate RNase H. They showed reduced anti-HIV activity compared to PS-oligos [17]. These results suggested that for optimum activity, antisense oligonucleotides should have combination of various properties instead of only increased stability towards nucleases or high affinity to target RNA.

We have made attempts to combine two modified oligonucleotides in order to generate a mixed-backbone oligonucleotide (MBO) that brings together the beneficial properties of the two molecules [71,72,78,80–82,86,87]. MBOs in general have two segments: one that contains an oligonucleotide analog capable of activating RNase H, and another that does not activate RNase H (Fig. 2). The oligonucleotides that activate RNase H are those bearing at least four contiguous phosphodiester or phosphorothioate

*PS-Oligonucleotide*



*End Modified MBO*



*Centrally Modified*

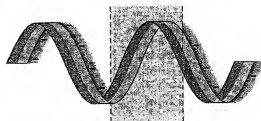


Fig. 2. Structure of PS-oligonucleotide and MBOs. In PS-oligos one of the non-bridging oxygen atoms in the phosphate backbone is substituted by a sulfur. In general, various biophysical and biochemical properties of PS-oligos are controlled by phosphate backbone nucleoside sugar, and heterocyclic bases. To modulate many of these properties, mixed-backbone oligonucleotides have been studied. MBOs refer to oligonucleotides which combine the advantages of two different modified oligonucleotides. Based on the position of incorporation of modified oligonucleotides in PS-oligos, MBOs can be classified as end-modified MBOs and centrally modified MBOs.

internucleotide linkages [80,81]. Of the oligonucleotide analogs that do not activate RNase H, we have studied oligodeoxynucleotides containing methylphosphonate [81-83,86,87], phosphoramidate [81,88], methylthiophosphonate [89], methylphosphotriester [90], methylphosphothiotriester [90], carbamates [91], and oligoribonucleotides containing 2'-*O*-methylribonucleosides [78], 2'-*O*-methylribonucleoside with methylphosphonate linkage [92,93], and 2'-*O*-methylribonucleoside with phosphoramidate linkages (D. Yu, S. Agrawal, unpublished data), and 2',5'-linked oligoribonucleotides [94].

The positioning of these analogs of oligonucleotides in a given sequence is crucial to the outcome and should be chosen carefully. We have studied two types of MBOs: end-modified and centrally modified MBOs.

### 10. End-modified MBOs

In end-modified MBOs, a non-RNase-H-activating analog of oligonucleotide is placed at the 3'-end or at both the 3'- and 5'-ends of the PS-oligo (Fig. 2). The purpose of incorporating various modified oligonucleotides in MBOs is to modulate biophysical, biochemical, or biological properties [71-73,78,80-82,86-94]. It has become evident that they also provide improvement in pharmacokinetic and safety profiles. The end-modified MBOs that have been studied extensively contain either methylphosphonate internucleotide linkages [81] or 2'-*O*-methyloligoribonucleosides [78].

End-modified MBOs, in general, have increased in vivo stability due to their increased resistance towards nucleases [78,95]. Because of this feature, some of the end-modified MBOs have shown good bioavailability following oral or colorectal administration [96,97]. The advantage of increased in vivo persistence of MBOs over PS-oligo is that it may allow less frequent administration for pharmacological activity.

Appropriate placement of the modified oligo in an end-modified MBO can decrease the toxicity of PS-oligos [98]. Oligo 5 (Table 1) administered to rats showed a toxicity profile similar to that of other PS-oligos; alteration or modification of the CpG dinucleotides reduced the toxicity (oligos 6, 7, and 8,

Table 1 and Fig. 3B). Incorporation of four 2'-*O*-methylribonucleosides at both the 3'- and 5'-ends of oligo 5, in which the CpG motif was also modified, produced oligo 9, which showed reduced toxicity (Fig. 3B). Incorporation of four 2'-*O*-methylribonucleosides at both the 3'- and 5'-ends of oligo 5, in which the CpG motif was not modified, produced an increase in toxicity [60]. An improved safety profile was also observed with an end-modified MBO (oligo 13) of oligo 10. (Fig. 2) [97]. The end-modified MBOs in both cases showed similar or improved biological activity. Oligo 13 is presently in Phase I human clinical trials and have shown overall improved safety profile including complement activation, prolongation of aPTT and thrombocytopenia [99].

End-modified MBOs also produce fewer polyanionic-related side effects than do PS-oligos [71, 72]. To further minimize the polyanionic characteristics of PS-oligos, attempts have been made to reduce the number of phosphorothioate linkages in MBOs by incorporating 2'-*O*-alkylribonucleosides along with a phosphodiester backbone [79]. Incorporation of 2'-*O*-methyloligoribonucleotides containing phosphodiester linkages at both the 3'- and 5'-ends of the PS-oligo failed to provide nuclease stability comparable to that of the PS-oligo [79]. Similar results have been observed with bulkier 2'-*O*-alkyl groups, including 2'-*O*-propylribonucleosides [58]. Recently, MBOs containing 2'-*O*-methoxyethoxyribonucleosides have been studied, but there is no report of in vivo stability of these MBOs yet [100]. We recently reported that in end-modified MBOs, the number of phosphorothioate linkages can be reduced by introducing alternative phosphodiester and phosphorothioate linkages in a 2'-*O*-methylribonucleoside segment without compromising the nuclease stability [101]. These end-modified MBOs have produced significantly less prolongation of aPTT [101], suggesting that by careful balance, the number of phosphorothioate linkages can be reduced to minimize protein binding without affecting in vivo disposition.

### 11. Centrally modified MBOs

In centrally modified MBOs, the modified oligonu-



in of four 2'-O-3'- and 5'-ends was also modified reduced toxicity. 2'-O-methylribonucleosides of oligo 5, in addition, produced an altered safety profile. The modified MBO (oligo 5) had an end-modified similar or improved safety profile. Presently in Phase I clinical trial, overall improvement in thrombocytopenia

to induce fewer polydeoxy PS-oligos [71], anionic character has been made to re-evaluate linkages in cytosolic nucleosides [79]. Incorporation of nucleosides containing the 3'- and 5'-ends improved nuclease stability [79]. Similar results with bulkier 2'-O-alkyl nucleosides [58]. 2'-O-methoxyethoxy, but there is no evidence of MBOs yet [100]. d-modified MBOs, linkages can be re-phosphodiester and 2'-O-methylribonucleosides. The modified MBOs have a portion of aPTT [101], i.e., the number of platelets reduced to minimizing in vivo dispo-

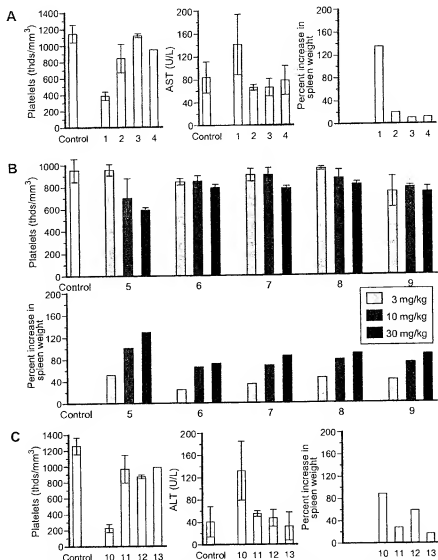


Fig. 3. Toxicity of oligonucleotides in mice and rats. (A) Oligos 1 to 4 were administered intravenously to CD-1 mice at a dose of 30 mg/kg daily for 7 days. On day 8, mice were killed and their spleens removed and weighed. Blood samples were taken for platelet counts and levels of serum aspartate aminotransferase (AST). Oligo 1 caused a decrease in platelet count and an increase in AST levels and spleen weight. Modification of the CpG motif in oligos 2, 3, and 4 resulted in minimization of these side effects. (B) Oligo 5 was administered intravenously to Fischer-344 rats at doses of 3, 10, and 30 mg/kg daily for 7 days. On day 8, the rats were killed and their spleens removed and weighed. Blood samples were taken for platelet counts. Oligo 5 caused a dose-dependent decrease in platelet count and an increase in spleen weight. Modification of the CpG motif in oligos 6, 7, 8, and 9 had some minimization of these side effects. (C) Oligos 10 to 13 were administered intravenously to CD-1 mice at a dose of 30 mg/kg daily for 7 days. Blood samples were processed by the same procedure as in the case of A. Oligo 10 caused a decrease in platelet count and an increase in spleen weight compared to oligos 11, 12, and 13, suggesting that flanking sequence and site of CpG motif in PS-oligos are critical for its impact on toxicity. For details of the above protocol, please refer to Agrawal et al. [60].

deotide is incorporated in the center of the PS-oligo [82,83]. The main advantage of centrally modified MBOs over PS-oligos is that they have few polyanionic-related side effects because they have shorter

segments of PS-oligos [82,83]. In addition, oligonucleotides (including 2'-O-alkylribonucleotides) containing phosphodiester linkages can be incorporated. Because of the presence of PS-oligos at both

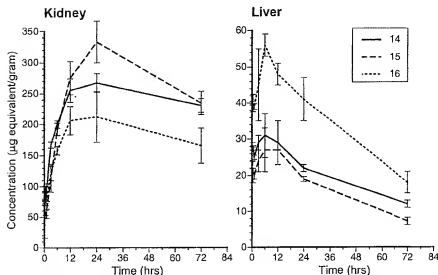


Fig. 4. Impact of number of phosphorothioate linkages on tissue disposition. Oligos 14, 15, and 16 were administered to mice at a dose of 30 mg/kg, intravenously. At various time points, animals were killed and various pharmacokinetic parameters were studied (see [83] for details). Disposition of oligo 14, 15, and 16 in kidney and liver showed that oligo 14 and 15, which have reduced phosphorothioate linkages, had increased accumulation in kidney and decreased accumulation in liver compared to oligo 16 which is completely phosphorothioate. Also, increased urinary elimination was observed with oligos 14 and 15 compared to oligo 16 [83].

3'- and 5'-ends, the centrally modified oligonucleotide region is not exposed to exonucleases and therefore remains intact [83]. We have studied centrally modified MBOs containing methylphosphonate [82,83] and 2'-*O*-methylribonucleosides containing phosphorothioate and phosphodiester linkages, and have observed improvements over PS-oligos in many respects [82,83].

In general, MBOs have provided encouraging results over PS-oligos and are being explored for their therapeutic potential in human clinical trials [99]. Other modifications are being explored as well, to further improve the therapeutic potential.

## 12. Structural modification of PS-oligos

To improve the nuclease stability and minimize the polyanionic nature of oligonucleotides, self-stabilized oligonucleotides have been developed [102,103]. These self-stabilized oligonucleotides are PS-oligos that contain a hairpin structure at the 3'-end. This hairpin structure gets destabilized in the presence of target RNA, thereby allowing the antisense oligonucleotide to bind to target mRNA. Self-stabilized oligonucleotides have been studied for their biophysi-

cal, biochemical, and biological activities [102,103], and their pharmacokinetic [104] and safety profiles [61].

## 13. Bio-reversible analogs of PS-oligos

To improve cellular uptake and minimize undesirable polyanionic-related side effects, we have synthesized and studied PS-oligos that have been derivatized by attaching an acyloxyalkyl group to the internucleotide sulfur moiety; these are referred to as pro-drugs of oligos [105–107]. Incubation of pro-drugs of oligos with esterase bio-reverts the pro-drug to the parent PS-oligo. The rationale behind the use of a pro-drug is that by using the appropriate parent ester group, it would be possible to design pro-drugs for sustained release, site-specific targeting, and oral bioavailability, in addition to above mentioned advantages.

## 14. Delivery of oligonucleotides

It is important to note that while the sequence of oligonucleotides and its modification are important

factors in a tides, includ elimination cacy in viv diester inter following th have increa: major tissu ing 2'-*O*-n thioate link: imals simila significant ir Binding of reservoir, sa nation of PS which affect: has been sho Reduction ii production c phonate) or (e.g., 2'-*O*-m increased dis to kidney ar By careful l thioate linka the rate of e sition can be l), which h were found l than was oli thioate linka tained with cleosides ar containing p 4). In addition administratio routes, includ gos, when ad good metabo extensive deg part of the g fied MBOs oc sides with ph taining methi showed imprc and lower par to PS-oligos.

factors in antisense activity, delivery of oligonucleotides, including tissue disposition, degradation, and elimination are also important factors for their efficacy *in vivo*. Oligonucleotides containing phosphodiester internucleotide linkages are rapidly degraded following their administration *in vivo* [52]. PS-Oligos have increased stability and are widely distributed to major tissues [51,52]. End-modified MBOs containing 2'-*O*-methylribonucleosides and phosphorothioate linkages have shown tissue disposition in animals similar to that observed with PS-oligos, and a significant increase in stability has been achieved [95]. Binding of PS-oligos to serum protein serves as a reservoir, saturation of which results in rapid elimination of PS-oligos in urinary excretion [51]. Aspirin, which affects PS-oligos by binding to serum proteins, has been shown to alter the pharmacokinetics [108]. Reduction in serum protein binding of oligos by introduction of non-ionic linkages (e.g., methylphosphonate) or with increased phosphodiester linkages (e.g., 2'-*O*-methylribonucleotides) has also resulted in increased disposition of administered oligonucleotide to kidney and elimination in urinary excretion [83]. By careful balance of the number of phosphorothioate linkages and other modified oligonucleotides, the rate of elimination and preferential tissue disposition can be achieved [101]. Oligos 14 and 15 (Table 1), which have fewer phosphorothioate linkages, were found less in the liver and more in the kidney than was oligo 16, which contained all phosphorothioate linkages [83]. Similar results have been obtained with PS-oligos containing 2'-*O*-propylribonucleosides and 2'-*O*-methoxyethoxyribonucleosides containing phosphodiester backbone [58,100] (Fig. 4).

In addition to parenteral route for oligonucleotide administration, we also explored non-parenteral routes, including oral and colorectal [96,97]. PS-Oligos, when administered to mice by gavage, showed good metabolic stability in the stomach; however, extensive degradation was observed in the lower part of the gastrointestinal tract [96,97]. End-modified MBOs containing either 2'-*O*-methylribonucleosides with phosphorothioate linkages or MBOs containing methylphosphonate internucleotide linkages showed improved metabolic stability in the stomach and lower part of the gastrointestinal tract compared to PS-oligos. Tissue disposition studies in mice

showed that end-modified MBOs were absorbed when administered by oral gavage and were distributed to major tissues. Similar results of absorption have been obtained following colorectal administration of end-modified MBOs [97].

The bioavailability of a drug is generally calculated based on the concentration of the drug in the plasma compartment versus time (area under the curve (AUC)) [109-111]. Oligonucleotides have short plasma residence times and are rapidly cleared to and retained by the tissues. The half-life of the oligonucleotide in plasma depends on serum protein binding and saturation, which may alter the distribution significantly. Based on our experience with various oligos, we have reached the conclusion that plasma pharmacokinetic parameters do not provide the whole picture in terms of bioavailability in the case of oligonucleotides. Concentration of oligonucleotides in tissues should also be considered when calculating the bioavailability.

## 15. Future directions

Rapid strides are being made in understanding the rules that govern the effective use of antisense oligonucleotides. It is clear that oligonucleotides can exert biological effects by multiple mechanisms, and the therapeutic potential of oligonucleotides can be explored based on these mechanisms. The sequence of an oligonucleotide is one of the key factors in controlling its mechanism of action and specificity. Selected oligonucleotide sequences can be appropriately modified to enhance the desirable properties and minimize the undesirable properties for their intended uses. Ongoing studies with second-generation oligonucleotide MBOs will further guide us in improving therapeutic potential of antisense oligonucleotides. These modified oligonucleotides which have specific mechanism of action can be used widely for gene target validation as well.

## Acknowledgements

I am grateful to past and present colleagues and collaborators who contributed to the work described in this review and whose names appear in the refer-

ences. I also wish to thank Ms. Shannon Gately for her expert secretarial assistance in processing this review.

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